

Accumulation of HLA-DM, a regulator of antigen presentation, in MHC class II compartments

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Abstract:

The HLA-DM genes encode an unconventional human leukocyte antigen (HLA) class II molecule that is required for appropriate binding of peptide to classical HLA class II products. DM may facilitate peptide binding to class II molecules within these intracellular compartments.

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The HLA-DM genes encode an unconventional HLA (human leukocyte antigen) class II molecule that is required for appropriate binding of peptide to classical HLA class II products. In the absence of DM, other class II molecules are unstable upon electrophoresis in sodium dodecyl sulfate and are largely associated with a nested set of peptides derived from the invariant chain called CLIP, for class II-associated invariant chain peptides. DMA and DMB associated and accumulated in multilaminar, intracellular compartments with classical class II molecules, but were found infrequently, if at all, at the cell surface. Thus, DM may facilitate peptide binding to class II molecules within these intracellular compartments.

Major histocompatibility complex (MHC) class II molecules consist of heterodimers of a \approx 34-kD α and a \approx 28-kD β chain that interact with a third glycoprotein, the invariant chain (Ii) in the endoplasmic reticulum (ER). This complex is transported out of the ER through the Golgi stacks as a nonamer ($\alpha\beta Ii$)₃ (1). Invariant chain is then proteolytically removed and class II molecules accumulate in an acidic, lysosome-like compartment, termed MIIC in B lymphocytes (2). MIIC is placed late in the endocytic route and is a potential site for peptides derived from exogenous antigens to associate with class II molecules. Similar compartments have since been characterized in other cell types (3-6).

Insight into how peptides bind (that is, get loaded onto) class II molecules was provided by a series of cell lines with mutations in genes in the class II region of the MHC (7, 8). These cell lines have three interesting properties: (i) class II molecules on the cell surface are not recognized by certain conformation-specific antibodies, such as the DR3 antibody 16.23; (ii) upon polyacrylamide gel electrophoresis (PAGE) in SDS, class II heterodimers are less stable than those of normal cells and dissociate into their constituent polypeptide chains; and (iii) the mutant cells are defective in the presentation of protein antigens to T cells (9). All three phenotypes of the mutant cells are the result of mutations in either of the linked DMA and DMB genes (7, 8). In addition, another mutant cell line with a large deletion encompassing the DM locus (T2-DR3) develops abnormal MIIC and has class II molecules predominantly associated with the class II invariant chain-associated peptides (CLIP) (10, 11).

To investigate further the function of DM, we made rabbit antisera; FS2 recognizes recombinant DM α chain protein and AK3 recognizes the derived COOH-terminal peptide of the DM α chain. To demonstrate the specificities of the antisera, we used glycoprotein preparations from the B lymphoblastoid cell line LCL721, the deletion mutant 21.174, and the cell line 7.9.6, which bears a point mutation in DMB (7, 9, 12). Both antisera recognized specific bands on protein immunoblots of 33 to 35 kD and 30 to 31 kD, for antibody to DMA (anti-DMA) and anti-DMB, respectively (Fig. 1, A and B). (Fig. 1A and B omitted) These values are consistent with the predicted molecular sizes of 26 kD given that the DMA sequence contains two putative N-linked glycosylation sites and DMB, one (13). As additional specificity controls, we demonstrated that anti-DMA did not cross-react with conventional class II molecules expressed in L cell transfectants and identified unique spots on two-dimensional SDS-PAGE (14).

The sequence similarities between DM and the classical class II genes, their locations adjacent to one another in the class II region, and the identical phenotypes of DMA and DMB mutants all suggest that the DM α and β chains associate to form a heterodimer. To confirm this, cells from the Burkitt's lymphoma cell line Raji were metabolically labeled for 20 min and lysed. Extracts were precipitated with the antiserum to DMA, FS2 (Fig. 2A). (Fig. 2A omitted) Immunoprecipitated protein was eluted from protein A-Sepharose and reprecipitated with either anti-DMA or anti-DMB. Reprecipitation with anti-DMA revealed bands at 30 and 33 kD. These were specific for DMA, and both were seen after metabolic labeling with ³⁵S for 5 min (15). The lower band is not present in appreciable quantities in the steady state (Fig. 1A) (16) and may represent an incompletely glycosylated form of DMA that is degraded in the ER. Reprecipitation with anti-DMB produced a band at 30 kD. No protein was isolated by AK3, when incubated with the peptide used for immunization. Thus, we showed that DMA and DMB associate and that this interaction takes place, as it does for classical class II molecules, early during biosynthesis. The association is not necessarily direct, and DMA and DMB could form a complex with other proteins.

Next we looked for expression of DM at the cell surface (Fig. 2B). (Fig. 2B omitted) Whereas DM in glycoprotein isolates was successfully biotinylated and could be immunoprecipitated with AK3, little if any was detected with the same antibody after biotinylating only cell surface proteins. DR was precipitated in large quantities from both preparations. These data suggest that unlike the classical class II molecules, DM is found mainly intracellularly.

To further define where in the antigen processing pathway DM operates, we decided to localize it within the cell. Subcellular fractionation has recently been used as a means of analyzing class II antigen presentation (3-6). We used this technique to examine the steady-state distribution of DM in the melanoma cell line MelJuSo. Microsomes were separated in an electric field, and the positions of lysosomes (beta-hexosaminidase activity) and of the total protein peak were determined (Fig. 3A). (Fig. 3A omitted) The fractions isolated from MelJuSo were then analyzed by protein immunoblotting and stained with antibodies to class I molecules (to determine the position of the plasma membrane and of the ER), to class II molecules, and to DM. Class I and II molecules were distributed over the ER and the cell surface (fractions 16 to 26), whereas fractions 6 to 14 contained only class II molecules (Fig. 3B) (5). (Fig. 3B omitted) Fractions 6 to 14 also contained endocytosed horseradish peroxidase (HRP) and P-hexosaminidase activity (Fig. 3A) and corresponded to the position in the electric field of the recently defined class II compartment.

DMA was observed as a doublet in fractions 19 to 27, whereas in fractions 5 to 15 it mostly consisted of a slower migrating species (Fig. 3C). (Fig. 3C omitted) A similar distribution was seen for DMB (17). The peak of activity in these anodally migrating fractions mirrored that found for class II, as might be expected if the two molecules colocalized to one compartment. Fractions 18 to 28, where most of the

class II molecules were found, contained the ER and the plasma membrane. To define better the location of DM in these fractions, they were digested with endoglycosidase H (Endo H), which removes immature (high-mannose) N-linked glycans (Fig. 3D). (Fig. 3D omitted) Whereas DMA in fractions 6 to 16 was Endo H-resistant, the lower and major component of the DMA doublet in the other fractions (18 to 26) was sensitive to Endo H, indicating residence in the ER. The fainter upper band was unchanged in position after Endo H treatment and could represent a minor proportion of the total protein present at the cell surface or possibly the Golgi, although the Golgi, as defined by galactosyl transferase activity, has been shown in this system to migrate slightly further toward the anode (18). Thus, the bulk of DM in MelJuSo was divided between the ER and a compartment that cofractionates with class II-containing vesicles. Percoll density gradient fractionation of Raji cells gave a broadly similar picture (19).

We then did immunoelectron microscopy on ultrathin cryosections of Raji cells. This confirmed that both DMA and DMB were located in typical multilaminar class II compartments or MIICs together with the majority of intracellular HLA class II molecules (Fig. 4, A and B). (Fig. 4A and B omitted) The plasma membrane had no detectable labeling for either DMA (Fig. 4A) or DMB. The ER labeling was weak, probably because of the large ER surface area and consequent lower density of DM. Ultrathin cryosections of 721.174 cells, which lack DMA and DMB genes, had no labeling with anti-DMA or anti-DMB. Morphologically, the DMA- and DMB-positive MIIC in Raji resembled those described in other human B cells (2, 10). MIICs contain internal membrane vesicles and sheets and express lysosomal enzymes and membrane proteins (2, 10). The DM-positive MIIC in Raji cells were positive for cathepsin D and the lysosomal membrane proteins CD63 and LAMP-1 (Fig. 4C), but were negative for the cation-independent mannose 6-phosphate receptor (CI-MPR), which is present on late endosomes but absent from lysosomes. (Fig. 4C omitted) The localization of DM to MIIC-like structures was also observed in the Epstein-Barr virus (EBV)-transformed B cell line LCL721 and in human dendritic cells isolated from peripheral blood (20).

When the DM genes were first described, several features indicated that they were unconventional class II molecules that have a unique function. DM expression paralleled that of other class II genes--that is, it is constitutive in B cells and inducible with interferon gamma in other cells (13). Comparison of the membrane-proximal immunoglobulin-related domains of DM with those of other MHC sequences shows that they share almost as much amino acid identity with class I as with class II molecules (≈30%) and must have diverged at around the same time that class I and class II sequences split from each other, long before duplication of the main class II loci, DP, DQ, and DR. DM protein sequences do not contain a recognizable CD4 binding site. However, computer modeling based on the DR coordinates (21) indicates that the alpha 1 and beta 1 domains of DM may fold in a similar way to the classical class II molecules (22).

Data from mutant cell lines implicating HLA-DM in class II antigen processing suggests a number of possible models for its function. DM might act as a CLIP sink, efficiently binding CLIP so as to leave conventional class II molecules available to seek other ligands. Alternatively, DM could act: as a shuttle to deliver peptides into a compartment for interaction with class II. A third model invokes the concept of DM as a chaperone, tethering other class II molecules in the MIIC until antigenic peptide is bound. Our results show that DM accumulates in the intracellular class II-containing compartment implicated as the site of class II peptide loading. But whereas classical class II molecules reside principally at the cell surface, in the steady state little if any DM was present there. It is possible that DM does reach the cell surface and is then rapidly internalized. Consistent with this hypothesis is the presence of a Tyr-X-X-Leu (X, any amino acid) sequence in the cytoplasmic tail of DMB (23). This consensus sequence functions in a number of other proteins (including LAMP-1, which colocalizes with DM to the MIIC) as a signal for rapid internalization from the cell surface in clathrin-coated pits. The accumulation of DM in an intracellular compartment where loading of class II molecules probably occurs suggests that DM may be

directly involved in this process.

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14. Lysates from Aaji cells and from L cells expressing DQ, DP, or DR, as well as lysates from the untransfected GellS, were analyzed by SDS-PAGE and protein immunoblotting. Anti-DR sub alpha and anti-DP reagents (mouse mAb 1B5 and a polyclonal rabbit antiserum raised against purified DP, respectively) recognized protein in Raji lysates and in the lysate from the relevant transfected cell line. whereas FS2 reacted only with Raji and not with any of the transfected cells. Glycoproteins were prepared from Raji cells and separated by nonequilibrium two-dimensional gel electrophoresis. The second dimension was immunoblotted and the membrane probed with FS2 and with 1B5. These two reagents produced different and unique patterns of reactivity, again indicating that the FS2 reagent does not cross-react with DR sub alpha .
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16. On pulse-chase analysis of Raji cells. FS2 precipitated a 30- to 33-kD doublet immediately after a 20-min labeling period. The 30-kD component gradually disappeared over the next 2 hours, leaving a doublet at 33 to 35 kD.
17. Identical protein immunoblots were probed with AK3.

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19. Membranes were prepared from Raji cells with a ball-bearing homogenizer and separated on a Percoll density gradient. Fractions were analyzed as for the MelJuSo cells, with similar results. Here we have presented only the data from MelJuSo, because this system has previously been characterized and described (5).
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26. Anti-DMA (FS2) was raised against a truncated DMA product (amino acids 4 to 104 of the predicted mature protein sequence) produced as inclusion bodies in *Escherichia coli*, by use of a T7 expression system (24). Rabbit anti-DMB (AK3) was raised against the COOH-terminal 15 amino acids of the molecule (13). AK3 was purified according to a standard protocol (25). Blots were developed through the use of enhanced chemiluminescence (Amersham).
27. Raji cells were starved for 90 min in cysteine- and methionine-free medium followed by metabolic labeling for 5 min with [³⁵S]methionine and [³⁵S]cysteine and lysed in 1% NP-40 lysis buffer. Immunoprecipitation with FS2 was carried out essentially as described (15) except that before addition of the antiserum the postnuclear supernatant (PNS) was denatured by boiling for 5 mins in 2K SDS and then diluted 10X in NP-40 lysis buffer. Analysis by SDS-PAGE revealed a doublet with bands at 30 and 33 kD.
28. Raji cells (7×10^7) were metabolically labeled for 20 min as described (15). The cells were lysed in 0.5% NP-40 lysis buffer and immunoprecipitated with FS2. The immunoprecipitate was eluted off protein A-Sepharose beads by boiling in 2% SDS, 0.5 mM dithiothreitol, 20 mM Tris (pH 7.4) for 3 min. Before reprecipitation, the eluate was diluted 10-fold in NP-40 lysis buffer.
29. Sulfosuccinimidyl-6-(biotinamido)hexanoate was used at a concentration of 0.1 mg/ml and incubated either with the glycoprotein preparation (0.7 mg/ml) or with whole cells in phosphate-buffered saline (5×10^6 /ml) for 1 hour at room temperature. The reaction was stopped by the addition of glycine to 10 mM. Biotin was removed from glycoproteins by spinning through a microfiltration device (Amicon). Immunoprecipitation was carried out as described (15) except that the antibodies were prebound to protein A-Sepharose.
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